

EFFECTS OF DAILY ORAL INJECTIONS OF QUERCETIN ON IMPLANTED
COLON-25 TUMOR GROWTH IN BALB-C MICE

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The effects of three oral dosages (0.4 mg, 0.8 mg, and 1.6 mg) of quercetin on Colon-25 tumors implanted in Balb-c mice were studied. The data in this study show that: (1) certain dosages of quercetin in alcohol solutions, reduces the weight, and size of implanted Colon-25 tumors in Balb-c mice, (2) these same dosages of quercetin all produce a profound neutrophilia combined with a significant lymphopenia at day 20 post-implantation, and (3) there was relatively little evidence of histological changes in the quercetin-treated tumor section which would indicate that the action(s) of quercetin is primarily at the subcellular level probably within the nuclei of the tumor cells.

Acknowledgments

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CHAPTER I

INTRODUCTION

In his text, Boik (1995) reviewed the reported biological actions of a certain group of natural substances called bioflavonoids of which quercetin is a member. Research on these flavonoids began in the 1930's in the laboratory of Szent-Gyorgyi, the discoverer of vitamin C (Bruckner and Szent-Gyorgyi 1936). They are found in a variety of foods (citrus fruits, vegetables) as well as beverages (coffee, tea, wine). Boik divided the flavonoids into five categories (anthocyanins, minor flavonoids, flavones or flavonoids, isoflavonoids, and tannins). Quercetin is a member of the flavones group that are thought to be the most widely distributed. Over the past 60 years, bioflavonoids have been reported to be involved in several important biological processes including: (1) antiallergic reactions (2) immunological modulation (3) the clotting sequence involving platelet aggregation and (4) antitumor actions.

This study was based on the reported antitumor properties of quercetin that include: lymphocyte proliferation (Pignol et al. 1988), neutrophilia (Musiani et al. 1996), free radical scavenging (Affany et al. 1987), angiogenesis (Teicher et al. 1991), the mitotic cycle in tumor cells (Yoshida et al. 1990), altering gene expression (Kioka et al. 1992), and inducing the phenomenon of "cell suicide" (apoptosis) in cells (Indap et al. 1997).

Most of the work done with quercetin has been concerned with its effects on various cancer cell cultures *in vitro*. The literature is relatively sparse with data from the

in vivo experiments involving intact, solid tumors in mammals under controlled laboratory conditions.

The general aim of this study, therefore, was to measure the effects of varying dosages of quercetin injected over a controlled time period on the growth of implanted Colon-25 tumor brei in immunodeficient Balb-c mice.

The specific parameters that were measured included: (1) changes in growth rate (2) tumor size and weight (3) tumor histology and (4) leucocyte differential count in the mice.

The data obtained in this study should provide information on the feasibility of the use of quercetin as an adjunct to the cancer therapy protocols in current use.

CHAPTER II

MATERIAL AND METHODS

Twenty-five male and twenty-five female mice, ranging in weight (16-27g) and age (2-4 months) were bred at the University of North Texas Animal Care Facility. They were implanted with a 4mm x 4mm medullary section (brei) of a human Colon-25 tumor from a donor mouse. Implanted sections were measured using a Petri dish with a piece of 1mm x 1mm graph paper attached underneath (see Figure 1A). Excised tumors were first placed in a Ringers solution to insure their viability after their removal from the donor mouse. The moistened 4mm x 4mm section was then injected between the iliac crest and the ribcage posteriorly below the epidermis using a 13 gauge needle. This strain of mice was used because of its reduced immune system, thereby facilitating the growth of implanted tumor brei into solid tumors within days.

The mice were maintained at the University of North Texas Animal Care Facility in a temperature regulated room and on 12 hour light-dark cycle. They were given water ad libitum, and fed a diet of Teklad rodent pellets.

The quercetin was donated by Thorne Industries (P.O. Box 25 Dover, Idaho 83825), and was analyzed for its purity by Freeman Industries (100 Marbledale Road Tuckahoe, New York 10707-0415).

Formulation of the Test Solutions

One gram of quercetin was combined with 312.5 ml of ethyl alcohol resulting in a stock solution with a concentration of 3.2 mg/ml. This concentration is the published

LD₅₀ for a 20g mouse (Stecher et al. 1960). The stock solution was diluted with distilled water prior to injection to avoid precipitation of quercetin. For the 1.6 mg/ml dilution, stock solution and distilled water were mixed in equal parts. The 1.6 mg/ml solution was again mixed with equal parts of distilled water to produce a 0.8mg/ml dilution. Finally, the 0.8mg/ml solution was mixed with equal parts of distilled water to produce a 0.4mg/ml dilution. This solution was administered orally five times per week (Monday-Friday) using a one ml plastic tipped pipette. The daily injections were given at or before noon, and measurements of the tumor using calipers (Figure 1B) commenced when the tumor could first be palpated (usually 7 days post-implantation). The quercetin injections were begun on the first day of palpitation and ceased on day 20 post-implantation. At this time, the mouse was euthanized by cervical dislocation, and the tumor was excised and placed in a 2% formalin solution.

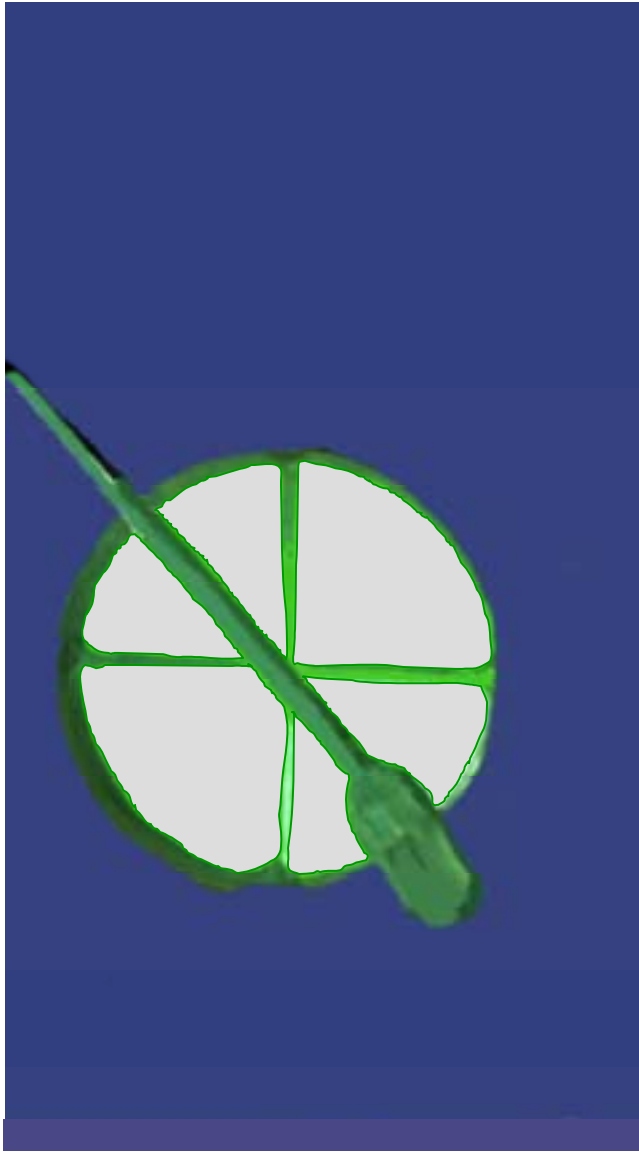
The mice were divided into control and test groups as follows:

- I. Control
 - A. Sham control animals received 1ml of distilled water (5 males and 5 females)
 - B. Control animals received 0.5ml of 98% ethyl alcohol in 0.5ml of distilled water (5 males and 5 females)
- II. Test animals (Quercetin)
 - A. 1.6mg quercetin (5 male and 5 female mice)
 - B. 0.8mg quercetin (5 male and 5 female mice)
 - C. 0.4mg quercetin (5 male and 5 female mice)

The parameters examined were: (1) daily changes in tumor size (length x width), (2) changes in blood leukocyte differential count using *Diff-Quick* differential stains, (3) changes in autopsied tumor weights (grams), (4) changes in autopsied tumor histology.

The tumors were measured daily Monday thru Friday following the first day of physical palpitation of the tumor. On the 20th day of post-implantation, the tumors were removed, weighed, and placed in a 2% formalin solution. Each tumor was then embedded in paraffin and sectioned using a 820 Spencer microtome (Buffalo, New York) into 16-20 μm sections. The sections were then stained using Harris Hematoxylin 3 and eosin, and photomicrographs were taken using a Zeiss microscope fitted with a 35mm Olympus camera loaded with Kodak Ektachrome Elite 200 exposure 35mm film.

Students' two-tail t-test was used to test the statistical significance of the data. This study was approved by the Institutional Animal Care and Use Committee at the University of North Texas.



A. Petri dish with 1mm x 1mm grid



B. Mechanical calipers cm/inches

Figure 1. Devices for measuring tumor implant (A) and size (B).

CHAPTER III

RESULTS

The data obtained in this study are presented in the form of time-course-curves in Figures showing daily changes in tumor size which are summarized in Tables. The data were statistically analyzed using Students' two tailed T-test. Photographs of an encapsulated tumor *in situ* and an excised decapsulated tumor are shown in Figure 2A. Photomicrographs of medullary sections of tumors from animals treated with varying dosages of quercetin were compared with those from non-treated tumors.

A typical encapsulated tumor *in situ* is shown in Figure 2A. The tumor is solid, well defined, and relatively symmetrical. Since the tumors were implanted in the back along the spine between the ribcage and the pelvis, they all showed growth posterior to the site of implantation. Figure 2B shows a typical excised tumor at day 20 post-implantation. At autopsy, there were no signs of metastasis in the thoracic and abdominal regions.

The data in Figure 3 compare the growth rate of tumors in mice injected with either water (sham-controls) or alcohol alone (solvent controls). No statistically significant differences were noted in the tumor growth rate between the alcohol and the water-injected mice. This was confirmed by comparing the slopes of the time course growth curves seen in Figure 3 and Table 1. Moreover, there were no significant differences in the tumor growth rates between the male and female animals.

As shown in Figure 4 daily dosages of 0.4mg/ml quercetin brought about a decrease in the tumor growth rates in both male and female mice. Tumor growth rates of



A. Encapsulated tumor *in situ*



B. Excised decapsulated tumor

Figure 2 Colon-25 autopsied tumors from Balb-c mice at 20 days post-implantation

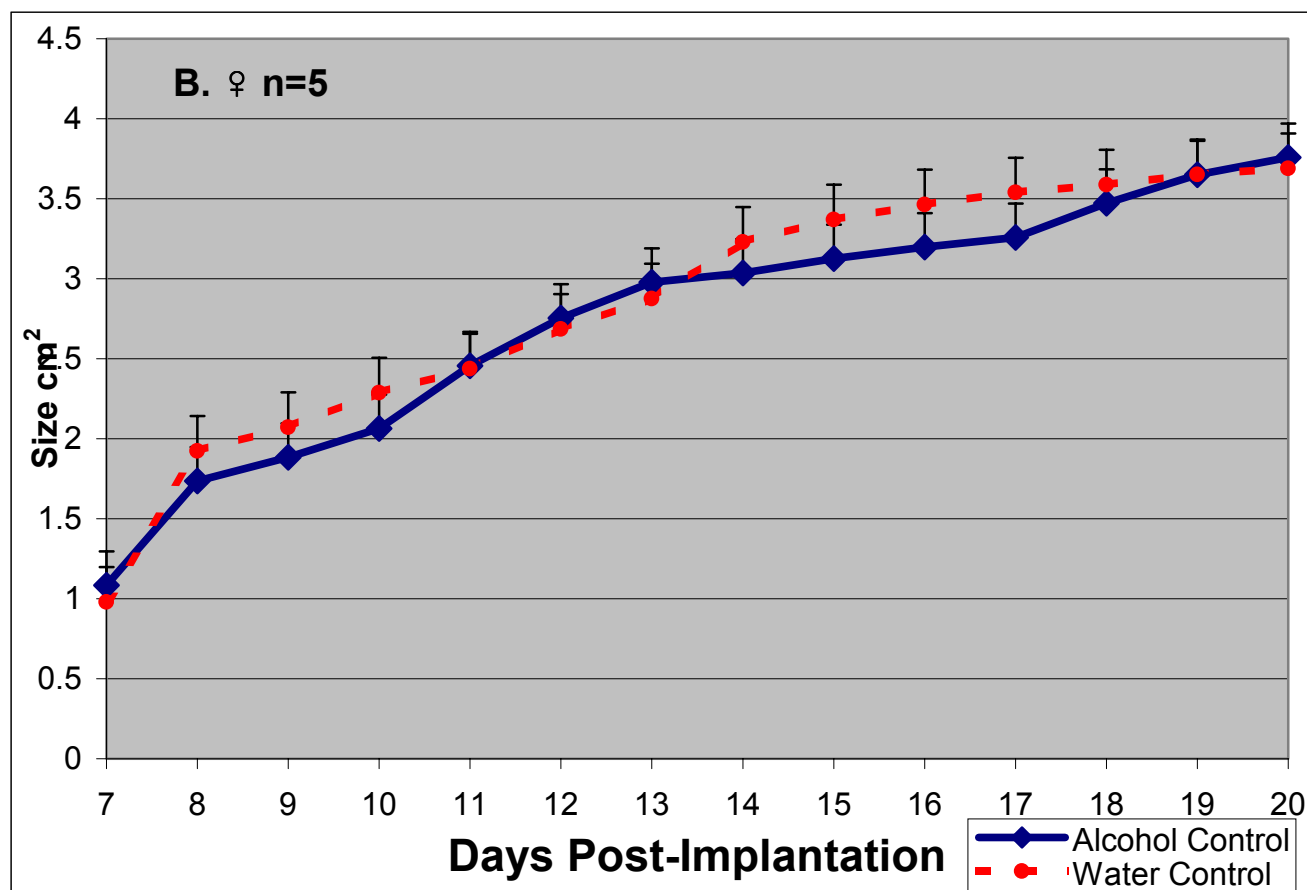
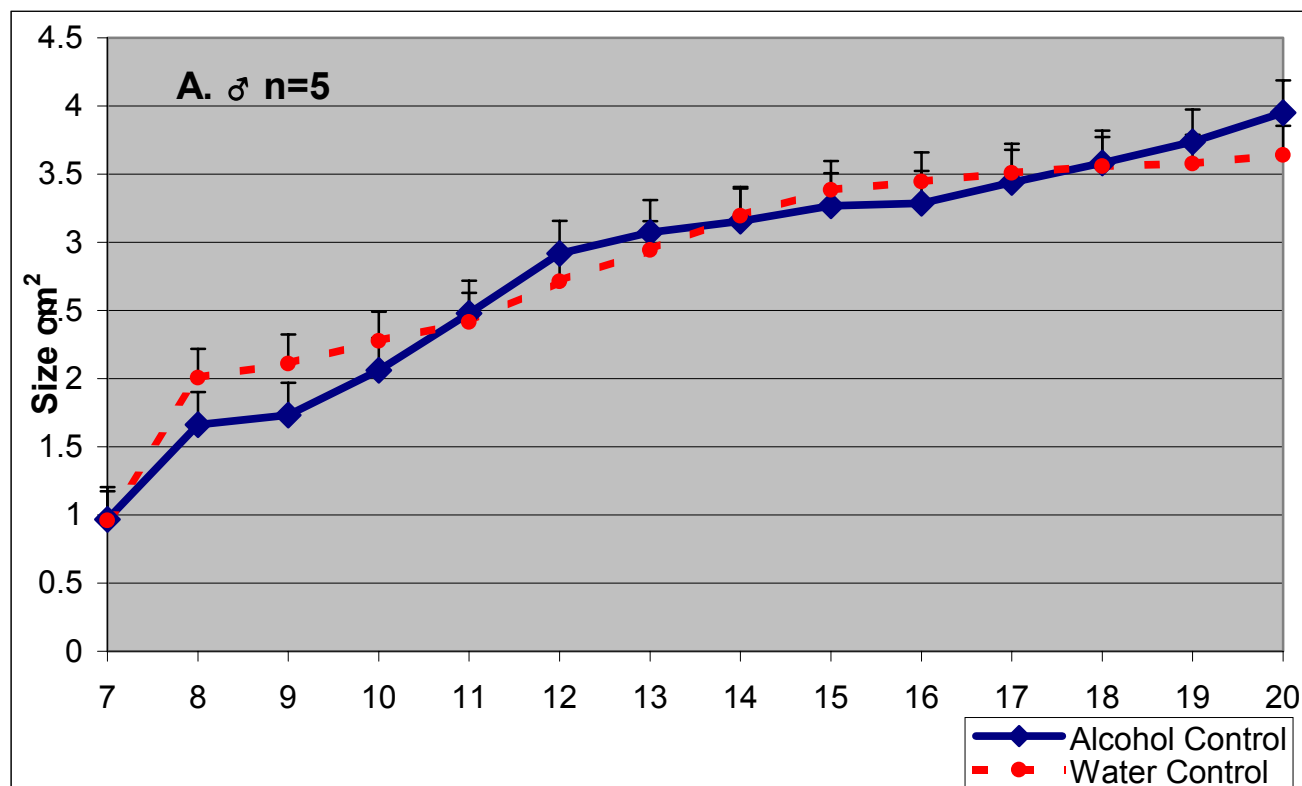


Figure 3 Effects of daily oral injections of alcohol and water on mean growth rates of implanted Colon-25 tumors in Balb-c mice. Day 7 represents initial time of measurable Δ tumor.

Length x Width					
<u>n</u>	<u>Dosage</u>	cm ²		SEM	%Δ
5	Water Control	2.84	±	0.65	0%
5	Alcohol-sham	2.81	±	0.60	-1%

A. Females

Length x Width					
<u>n</u>	<u>Dosage</u>	cm ²		SEM	%Δ
5	Water Control	2.99	±	0.56	0%
5	Alcohol-sham	2.87	±	0.54	-4%

$$\% \Delta = \frac{T - C}{C} \times 100$$

Statistical significance determined by a two tailed T test

Table 1 Summary of the effects of oral injections of water and alcohol on mean size of implanted Colon-25 tumors in Balb-c mice.

females were slightly higher than in males.

Figure 5 shows the mean growth rates of tumors in males (Figure 5A) and females (Figure 5B) treated with 0.8mg/ml of quercetin. Again, the tumors grew at a slower rate in both the treated male and female mice; growth of the tumors was slower in the males. The mean tumor growth rates, of male and female mice treated with 1.6mg/ml, are depicted in Figures 6A and 6B respectively. The abrupt drop in the growth curve in Figure 6A at day 18, was due to the death of a male mouse. A similar drop is evident in Figure 6B at day 14, when a female mouse died prematurely.

The effects of quercetin on Colon-25 mean tumor weight and size are summarized in Table 2. The only statistically significant change in tumor weight was observed at the 1.6mg/ml dosage level in males (56%)($t=1.10$ $p=0.02$). The other dosages yielded relatively the same degree of tumor weight loss in both the males and females (25-38%); these changes, however, were found to be statistically insignificant. All of the dosages of quercetin produced statistically significant decreases in tumor size. Tumor sizes in the males showed similar decreases (37-45%)(0.4 mg, $t=1.58$, $p<.001$; 0.8 mg, $t=1.73$, $p<.002$; 1.6 mg, $t=1.50$, $p<.001$) while the females showed similar but slightly higher decreases (36-50%)(0.4 mg, $t=1.40$, $p<.001$; 0.8 mg, $t=1.85$, $p<.002$; 1.6 mg, $t=1.43$, $p<.001$) in tumor size.

Figure 7 depicts the histological features in the autopsied tumors. In the alcohol-treated (Figure A) control sections, the tumor cells appear to be homogeneously distributed throughout the tissue without deterioration or increased vascularity. As shown in Figure 8B, some of the cells, from 0.4mg/ml treated tumors, appear to be distributed in elongated strands while other cells, appear to be slightly condensed. In the

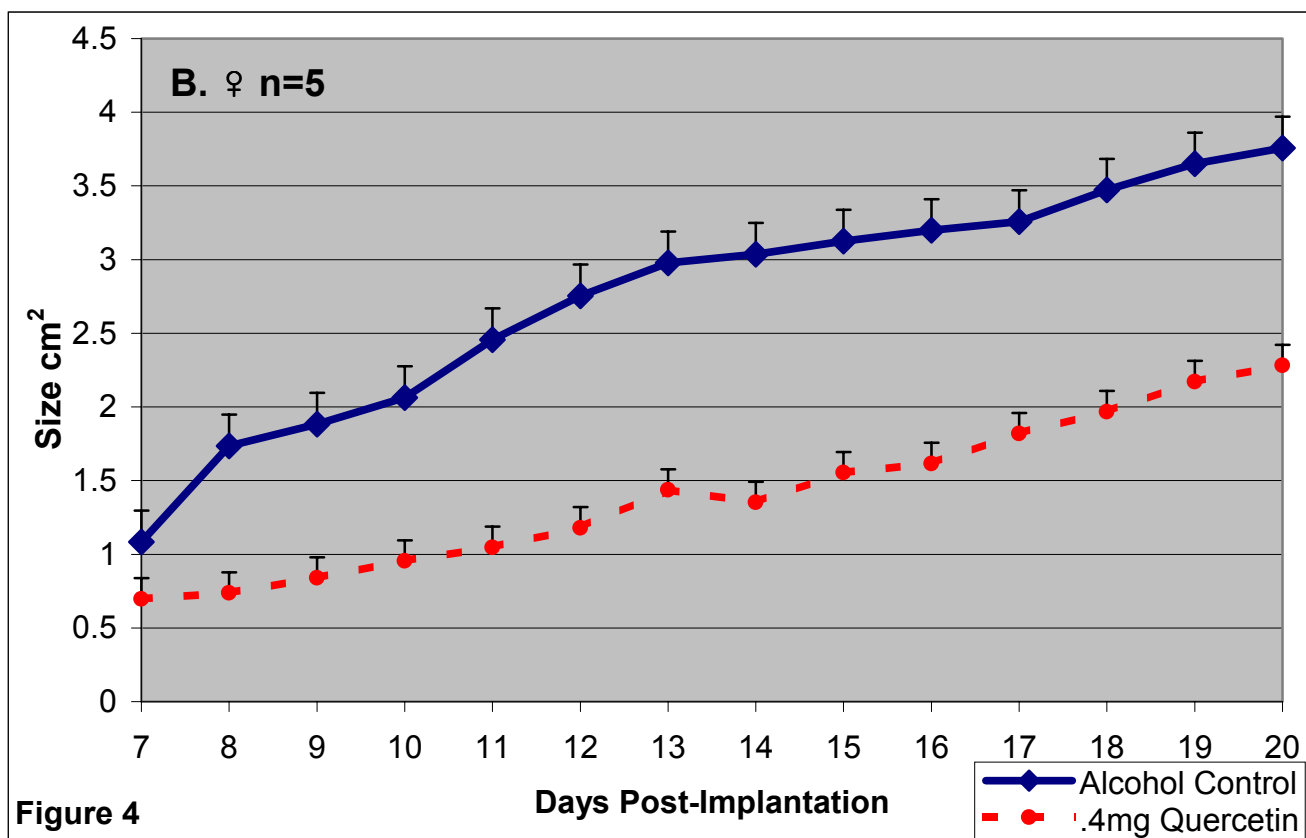
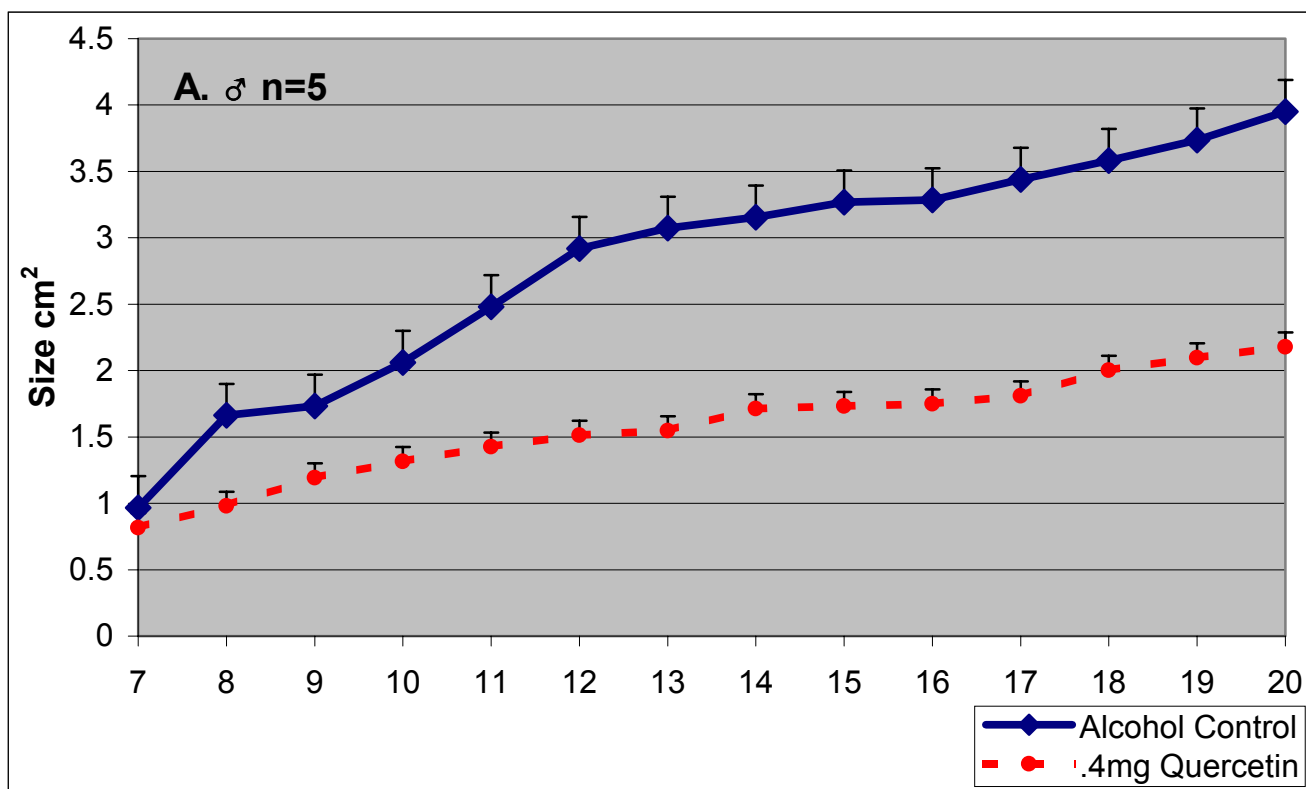


Figure 4

Effects of daily oral injections of 0.4 mg quercetin on mean tumor growth rates in Balb-c mice

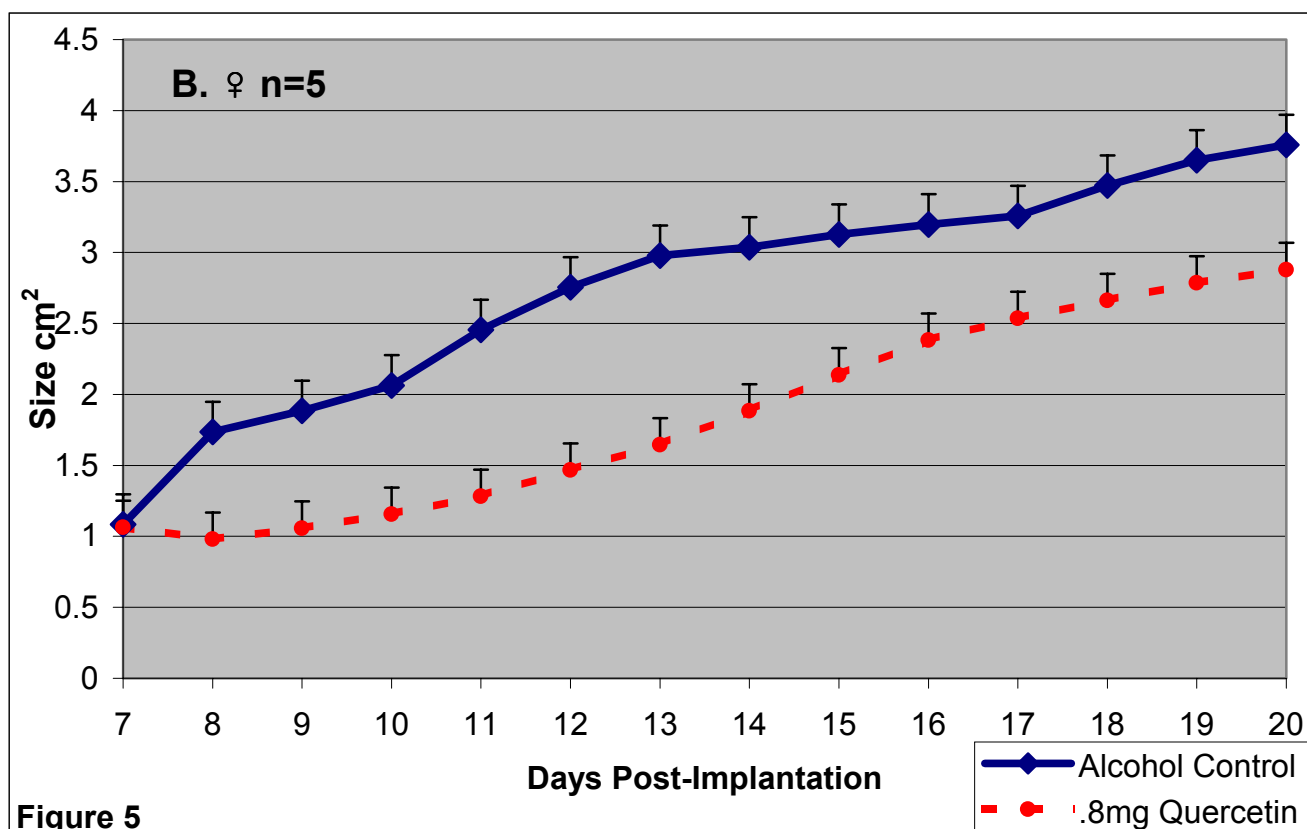
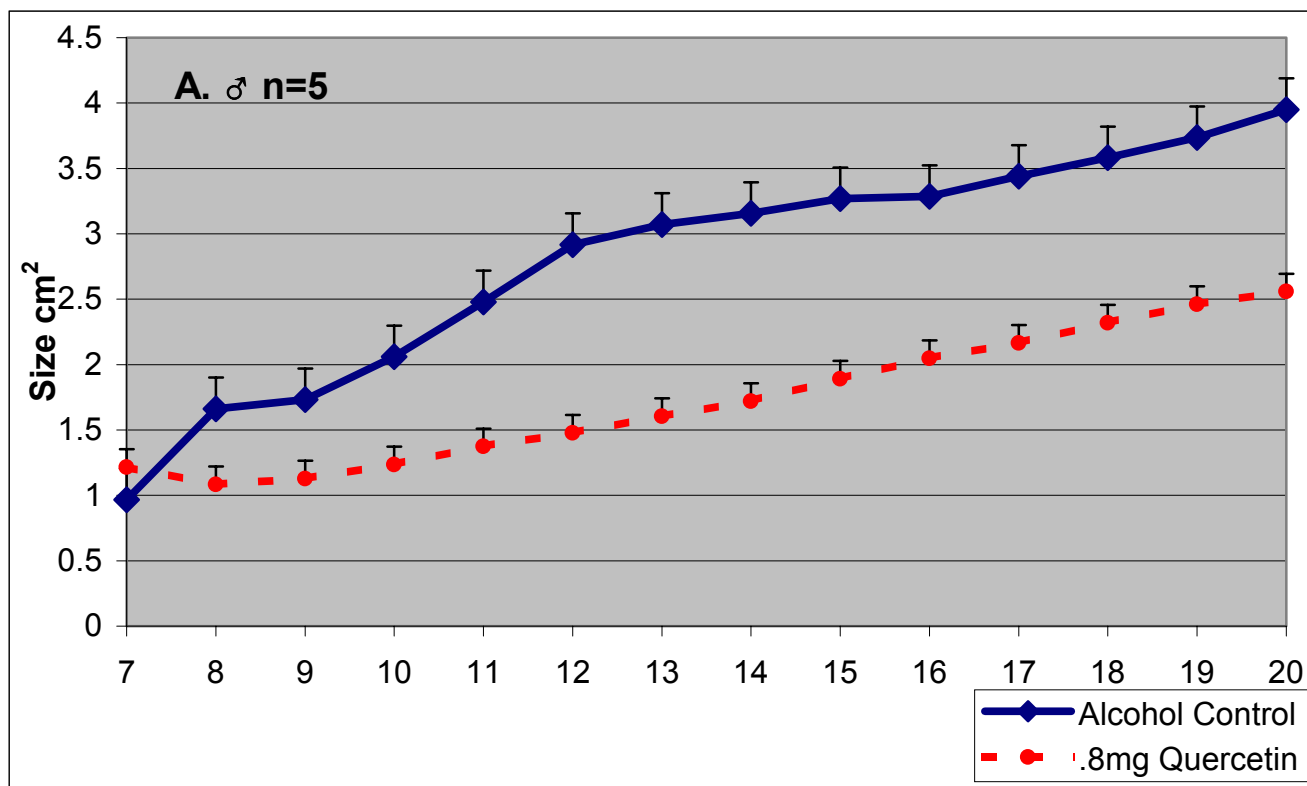


Figure 5

Effects of daily oral injections of 0.8 mg quercetin on mean tumor growth rates in Balb-C mice

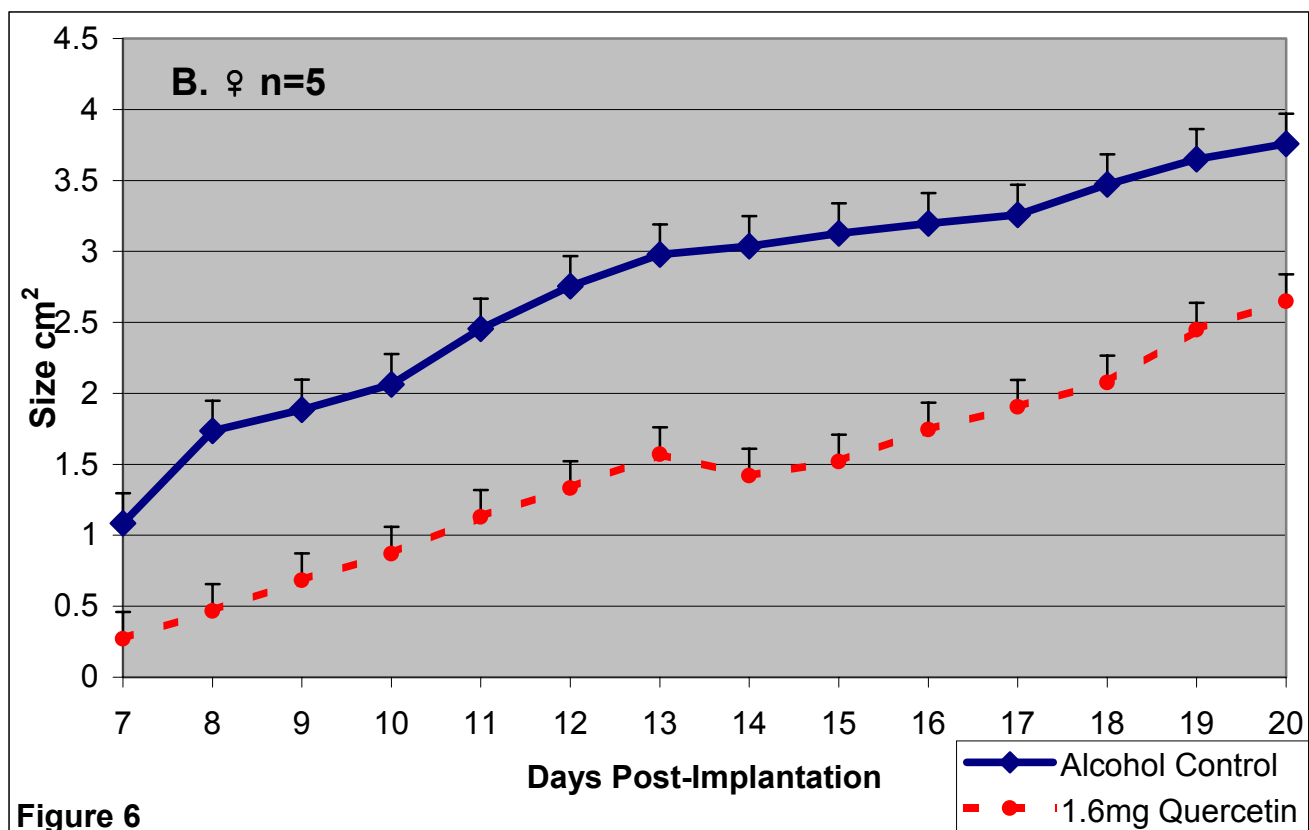
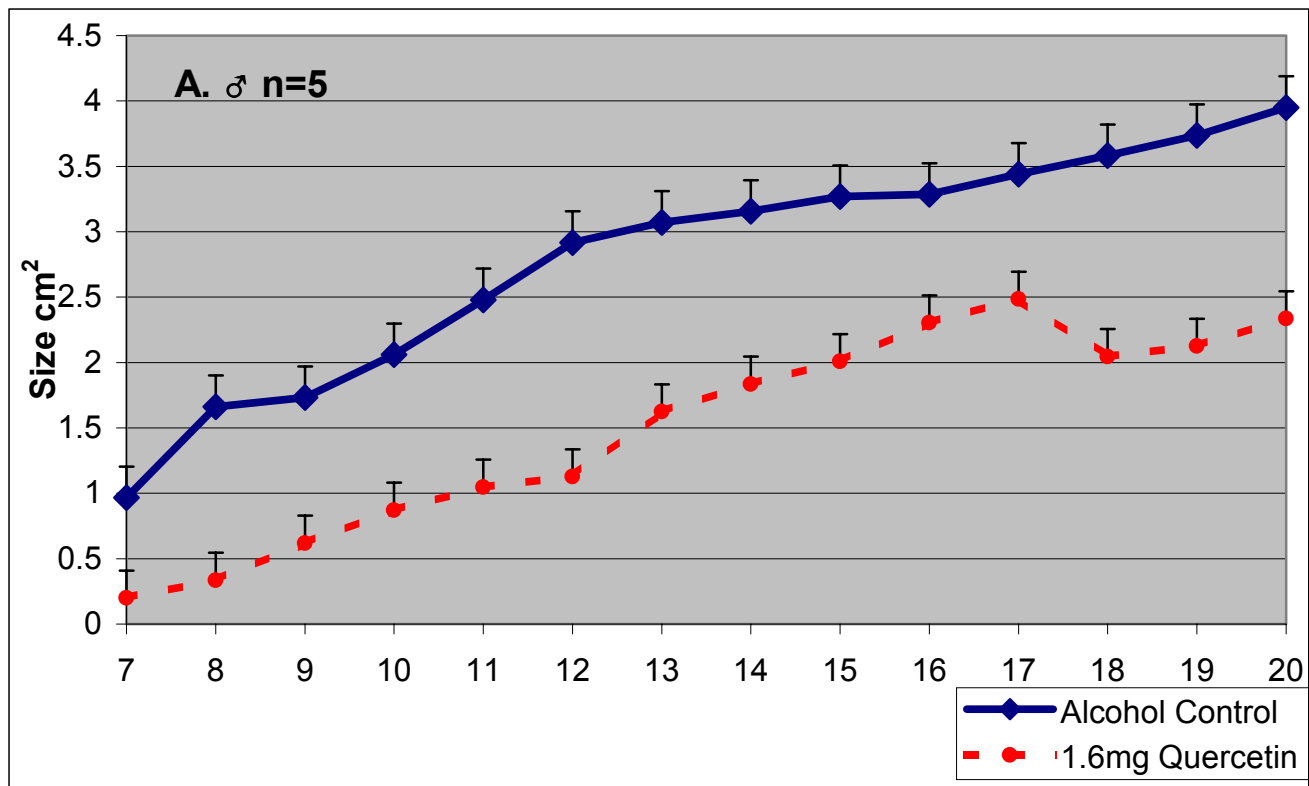


Figure 6

Effects of daily oral injections of 1.6 mg quercetin on mean tumor growth rates in Balb-C mice

A. Males

n	Dosage	Mean Wt.				Length x Width			
		grams		SEM	[%Δ] [*]	cm ²		SEM	[%Δ]
5	Alcohol-sham	2.488	±	0.92	0%	2.74	±	0.60	0%
5	0.4mg/ml	1.876	±	0.32	25%	1.58	±	0.32	42%***
5	0.8mg/ml	1.73	±	0.4475	30%	1.73	±	0.43	37%**
4	1.6mg/ml	1.1025	±	0.2125	56%*	1.50	±	0.68	45%***

$$^*\% \Delta = \frac{T - C}{C} \times 100$$

B. Females

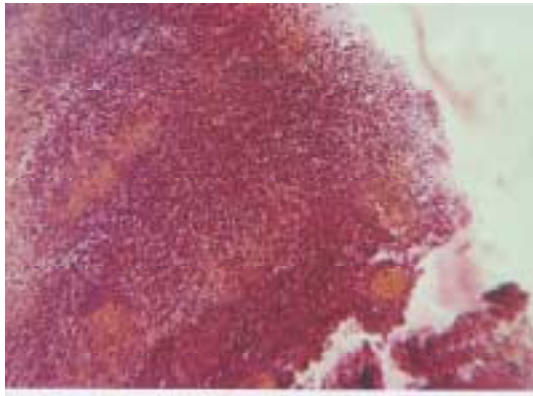
n	Dosage	Mean Wt.				Length x Width			
		grams		SEM	[%Δ]	cm ²		SEM	[%Δ]
5	Alcohol-sham	2.232	±	0.87	0%	2.87	±	0.54	0%
5	0.4mg/ml	1.448	±	0.32	35%	1.40	±	0.43	51%***
5	0.8mg/ml	1.374	±	0.4475	38%	1.85	±	0.62	36%**
4	1.6mg/ml	1.55	±	0.2125	31%	1.43	±	0.55	50%***

$$\% \Delta = \frac{T - C}{C} \times 100$$

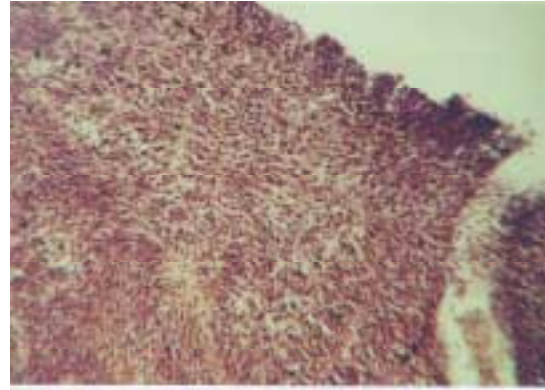
Statistical significance determined by a two tailed T test

- * Statistically significant @ p<.02
- * Statistically significant @ p<.002
- * Statistically significant @ p<.001

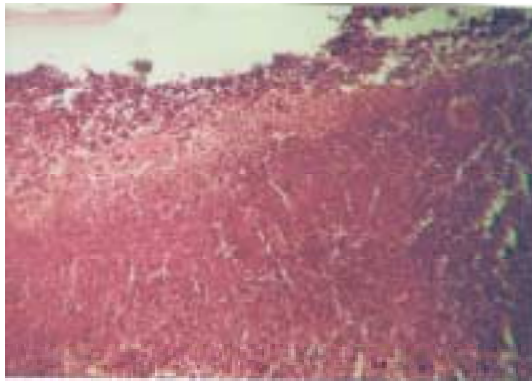
Table 2 Summary of the effects of quercetin on mean Colon-25 tumor weight and size at day 20 post-implantation in Balb-c mice.



A. Ethanol



B. 0.4mg/ml Quercetin



C. 0.8mg/ml Quercetin



D. 1.6mg/ml Quercetin

Figure 7. Effects of quercetin on the histology of autopsied Colon-25 tumors in Balb-c mice (x 100 Eosin-hematoxylin).

photomicrograph, Figure 8C, shows a section from tumors from the 0.8mg/ml quercetin treated animals. The cells in this section appear less homogeneously distributed and there is evidence of “crevicing” in the tissues. In the section of the tumor taken from mice receiving 1.6mg/ml quercetin (Figure 8D), the cells appear to be distributed in clusters and individual cells appear slightly enlarged with an increase in cell density (nuclear condensation?). In general, it was difficult to find any clear, well defined, differences between the histology of the sections of the treated and untreated tissues.

The effects of quercetin on the differential leukocyte counts are summarized in Table 4. The data show the change in the count/100 cells examined between the first day of implantation (T_0) to the 20th day post-implantation (T_{20}). Due to the paucity in the numbers of monocytes and basophils, it was difficult to establish statistical significance to the small changes that were observed. Interestingly, at day T_{20} there was observed distinct neutrophilia concomitantly with a distinct lymphopenia in all of the quercetin treated mice. The changes were statistically significant but were less pronounced at the highest dose level (1.6mg/ml).

<u>Dosage</u>		T ₀			T ₂₀			%Δ	
Sham	n	mean		SEM	mean		SEM		
	2	Neutrophils	19	±	1	15.5	±	5.5	-18%
	2	Lymphocytes	74	±	4.5	72.5	±	2.5	-2%
	2	Monocytes	5	±	3	2.5	±	1.5	-50%
	2	Basophils	2.5	±	0.5	0	±	0	-100%
0.4mg/ml									
	9	Neutrophils	19	±	1	61.9	±	18.6	229%***
	9	Lymphocytes	74	±	4.5	32.1	±	17.5	-57%*
	9	Monocytes	5	±	3	4.1	±	2.4	-18%
	9	Basophils	2.5	±	0.5	2.3	±	0.6	-9%
0.8mg/ml									
	7	Neutrophils	19	±	1	57	±	19.0	200%*
	7	Lymphocytes	74	±	4.5	36	±	21.1	-51%*
	7	Monocytes	5	±	3	4	±	1.1	-29%
	7	Basophils	2.5	±	0.5	4	±	1.6	60%
1.6mg/ml									
	5	Neutrophils	19	±	1	33.4	±	4.7	76*
	5	Lymphocytes	74	±	4.5	50.2	±	5.4	-32%
	5	Monocytes	5	±	3	10.6	±	7.8	112%
	5	Basophils	2.5	±	0.5	5.2	±	2.2	108%

$$* \quad \% \Delta = \frac{T_{20} - T_0}{T_0} \times 100$$

*p<.02, **p.002, ***p<.001

Statistical significance determined by a two tailed T test

T₀= initial count

T₂₀= count at day 20
post-implantation

Table 3 Summary of the effects of oral Quercetin on the mean WBC differential counts in Balb-c mice implanted with Colon-25 tumors.

CHAPTER IV

DISCUSSION

Three technical problems were encountered in this study: (1) the use of absolute ethanol as the solvent for the quercetin test solutions (2) the bioavailability of the quercetin given orally and (3) quantitation of the implants used.

Since the form of quercetin used was hydrophobic, it was necessary to use an established organic solvent ethanol. It was found that the tumor growth rates in the EtOH injected mice were almost identical to those receiving water only, indicating the absence of a separate EtOH action.

In regard to quercetin's bioavailability, the data are conflicting. In 1975 Gugler et al. found no measurable plasma concentration following oral administration of 4 grams of quercetin (Gugler et al. 1975). However, in 1995, Hollman reported that up to 24% of quercetin is absorbed in healthy human ileostomy volunteers. Probably the most relevant research came from Ueno et al. (1983). Through radioactive labeling, they were able to demonstrate the presence of quercetin in the liver, kidney, and blood, six hours after oral administration. They concluded that rats absorb approximately 20% of an oral dose, so it is probable that quercetin is also absorbed in mice. It is known that red wine, which is known to be high in quercetin, decreases one's chance of developing cancer; the possibility exists that an alcohol solution may enhance quercetin's absorption in the stomach.

To accurately quantify the amount of tumor implant, a grid was fixed underneath a Petri dish (Figure 1A) so that 4mm x 4mm sections (brei) could be measured and

implanted changes in tumor size (l x w) were measured daily with calipers following the first day of palpation, until the 20th day of post-implantation recorded in cm².

It was difficult to compare the results of this *in vivo* study with other *in vitro* studies which predominate the literature. However, several *in vitro* studies demonstrated growth inhibition of 50% or better in several cancer cell lines. Quercetin has been shown to inhibit leukemia and Ehrlich ascites tumors (Suolinna et al.1975), gastric (Yoshida et al.1990), and colon cancer (Hosokawa et al.1990) among other cell lines *in vitro* (Avila et al.1994). The data presented here support the *in vitro* studies, and suggest the presence of antitumor properties of quercetin on intact tumors in whole mammals.

What are the possible mechanisms involved with the anti-tumor actions of quercetin? Tumors require an increase in blood vessel vasculature (angiogenesis) in order to grow past 1-2 mm³ (Folkman 1990). Quercetin, like Folkman's "angiostatin", has been shown to interfere with angiogenesis in mice (Teicher et al. 1991). Quercetin is reported to do this by blocking enzymes at the membrane surfaces that are involved with the initial steps in angiogenesis (Levi et al. 1984).

In addition to a blood supply, tumors require optimal pH and cannot survive at a pH lower than 6.0 (Dobrowsky et al. 1991). Quercetin has been shown to inhibit lactic acid transport out of colon carcinoma cells, thereby lowering the intracellular pH (Agullo et al. 1994).

Heat shock proteins (HSP) protect cells against high temperatures as well as other stresses including radiation and chemical (Boik 1995). Quercetin has been shown to inhibit HSP induction in erythroleukaemia cells (Giuliano and Gabriella 1994) rendering cancer cells vulnerable to radiation and chemical treatment. Quercetin has also been used

in combination with radiation therapy, and protects the bone marrow of mice from radiation injury (Indap et al.1998). Quercetin, like other antioxidants, scavenges for free radicals which are often produced through radiation therapy, and have been known to cause tissue damage.

Quercetin has also been shown to be complementary to chemotherapy by inhibiting the expression of the multidrug resistance gene MDR1 in human carcinoma cells (Kioka et al. 1992). In addition to the MDR1 gene, quercetin has been shown to inhibit the mutant p53 gene in human breast cancer cell line MDA-MB468 (Avila et al.1994). The normal functioning p53 gene is known to trigger apoptosis in the controlling cell cycle; malfunctioning of this gene has been linked to neoplastic growth (Finnegan et al. 1994).

Many researchers now believe apoptosis to be a second type of cell death that is completely different from necrotic death. Unlike necrosis, apoptosis affects scattered, individual cells, does not produce inflammation, and thus does not damage adjacent normal cells. Apoptosis, therefore, is thought to represent a mechanism for removal of old, damaged cells in tumors. Perhaps, one cause of cancer is due to the failure of apoptosis to remove cells whose DNA has been damaged (Carson and Riberio 1993). Such failure might allow the pre-cancerous cell to progress from the initial stage of tumor production where the DNA damage is reversible to the proliferation stage where the DNA damage is permanent. It would seem that under some conditions of DNA damage; according to Schwartzman and Cidlowski 1993, Meyn et al. 1994 the cells may undergo apoptosis, rather than live to produce harmful mutations. There is some evidence that

certain cytotoxic anticancer drugs induce necrosis at high doses and apoptosis at low doses (Boik 1995).

Apoptosis, in which cells in effect commit suicide, has been observed in several *in vitro* experiments involving quercetin. The tell-tale signs of apoptosis include: morphological changes, loss of cell viability, and suppression of cellular progression through the mitotic cell cycle. In 1994, Wei et al. found morphological changes, condensation of the nuclear chromatin, and nuclear fragmentation in K562, Molt-4, Raji, and MCAS tumor cell lines treated with quercetin (Wei et al. 1994). Indap further confirmed Wei's work in 1997, when quercetin was reported to produce apoptosis in a dose-dependent manner with characteristic morphological changes such as membrane blebbing, nuclear condensation, and nuclear fragmentation (Indap et al. 1997). The nuclear fragmentation can be explained by quercetin's ability to actually produce DNA strand breakage (Rahman et al. 1992). Although the histological micrographs in this study did not reveal any signs of overt necrosis, evidence of nuclear swelling and/or nuclear condensation in quercetin treated animals was observed (Figures 8 B,C, & D). Thus, it may be that apoptosis was occurring in these tumors. Moreover, the observation that the quercetin-treated tumors grew at a slower rate, may indicate some kind of apoptotic action of quercetin.

Quercetin's actions on the general mitotic cycle (see below) have been reported by several workers (Havsteen 1983 and Yosida et al. 1990). Glycolysis is very important in dividing cells. It provides the energy in the form of ATP that the cells need to survive. Quercetin has been shown to inhibit the glycolytic pathway, in effect reducing a cell's ATP supply (Suolinna et al. 1975). This may be due, in part, to quercetin's selective

inhibition of certain glycolytic enzymes (lactate dehydrogenase and pyruvate kinase)(Grisiola et al.1975). In fact, quercetin was shown to be a competitive inhibitor of G type casein kinase and exhibited a high affinity for the ATP site of the enzyme (Cochet et al.1982).

Although the data presented do not reveal the precise mechanism(s) involved in quercetin action, one cannot deny quercetin's effect on tumor growth *in vivo*. While a dose-dependence action was not evident in the data presented here, quercetin's general effect was a significant (50%) reduction in tumor size (area). With such dramatic change in tumor size, one would have expected a similar decrease in tumor weight; however, the only significant case was in males treated with 1.6 mg/ml of quercetin which demonstrated a 56% reduction in weight. This observation might be due to the differences in the overall tissue constituents of the excised tumors (water vs. organic matrix).

One interesting observation in this study was a noticeable increase in the neutrophil count (neutrophilia) concomitant with a drop in the leukocyte count (lymphopenia) at 20 days post-implantation. This phenomenon was observed in previous unpublished experiments carried out by this lab. This "neutrophilia" has been observed in other Balb-c mice with mammary adenocarcinoma (Musiani et al. 1996). The drop in leukocyte count may be explained by quercetin's role as an antioxidant. Antioxidants, which are free radical scavengers, have been shown to decrease rat lymphocytes *in vitro* (Pignol et al. 1988).

In summary, the data in this study show that: (1) certain dosages of quercetin in alcohol solutions, reduce the growth rate, the weight, and the size of implanted Colon-25

tumors in Balb-c mice (2) these same dosages of quercetin all produce a profound neutrophilia combined with a significant lymphopenia at day 20 post-implantation and (3) there was relatively little evidence of significant histological changes in the quercetin-treated tumor sections, suggesting that the action(s) of quercetin is primarily at the subcellular level probably within the nuclei of the tumor cells.

Finally, according to Boik (1995) there are more than 135 different glycosides of quercetin remaining to be tested. The current study is being extended to include two of them quercetin-chalcone and quercetin pectin. The findings of this study suggest a clear need for a continued study of quercetin and the feasibility of it being a useful adjunct in certain cancer therapy protocols.

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